PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07F 9/38, A61K 47/48, C07K 14/00, A61K 31/66, 38/16 — A1

(11) International Publication Number: WO 96/24598

(43) International Publication Date: 15 August 1996 (15.08.96)

(21) International Application Number: PCT/CA95/00068

(22) International Filing Date: 10 February 1995 (10.02.95)

(71) Applicant *(for all designated States except US)*: THE UNIVERSITY OF BRITISH COLUMBIA [CA/CA]; Research Administration, IRC Building, Room 331, 2194 Health Sciences Mall, Vancouver, British Columbia V6T 1W5 (CA).

(72) Inventor; and

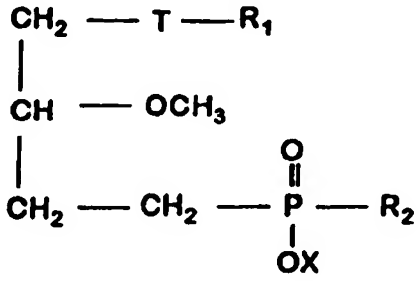
(75) Inventor/Applicant *(for US only)*: SALARI, Hassan [CA/CA]; 4677 Cannery Place, Ladner, British Columbia V4K 3X8 (CA).

(74) Agent: OYEN, Gerald, O., S.; Oyen Wiggs Green & Mutala, 480 - The Station, 601 West Cordova Street, Vancouver, British Columbia V6B 1G1 (CA).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, ARIPO patent (KE, MW, SD, SZ, UG), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: [4-HEXADECYL-3-METHOXY-BUTYL] PHOSPHONIC ACID AND ITS PROTEIN CONJUGATES USEFUL AS ANTI-CANCER AGENTS



(I)

(57) Abstract

This invention pertains to the synthesis of [4-hexadecyl-3-methoxy-butyl] phosphonic acid and its protein conjugates, and the use of these compounds as anti-cancer agents. A method of treating cancer in a mammal afflicted with cancer, comprising treating the afflicted mammal with a therapeutic amount of a phosphonic compound of formula (I), wherein T is an oxygen or sulphur atom, R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is a hydrogen atom, or a pharmaceutically acceptable cation, and either of the opposite stereochemical configurations [(R) or (S)], or a mixture of stereoisomers thereof, and pharmaceutically acceptable salts thereof.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia
AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CS	Czechoslovakia
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgystan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakhstan
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam

[4-HEXADECYL-3-METHOXY-BUTYL] PHOSPHONIC ACID AND ITS PROTEIN CONJUGATES USEFUL AS ANTI-CANCER AGENTS

FIELD OF THE INVENTION

This invention pertains to the synthesis of [4-hexadecyl-3-methoxy-butyl] phosphonic acid and its protein conjugates, and the use of these compounds as anti-cancer agents.

BACKGROUND OF THE INVENTION

European Patent No. P0230 575A2, dated April 12, 1986, discloses a group of glycerophospholipid compounds having an alkyl chain of C2-C22 and a methoxy group at the sn-2 position and a phosphocholine at the sn-3 position. These compounds are stated to be useful as anti-cancer agents.

U.S. Patent No. 4,408,052, dated February 25, 1981, assigned to Takeda Chemical Industries, Osaka, Japan, claims a group of phospholipid carbamates useful as anti-tumor agents.

Canadian Patent No. 1,248,534, dated January 10, 1989, granted to Takeda Chemical Industries of Japan, protects a group of ketolyso phospholipids, which purportedly are useful as antitumor agents.

U.S. Patent No. 4,515,722, dated May 7, 1985, granted to Merck Sharp & Dohme, protects a group of phosphatidylinositol analogs which are evidently effective in inhibiting phospholipase C and thereby have utility as anti-inflammatory and analgesic agents.

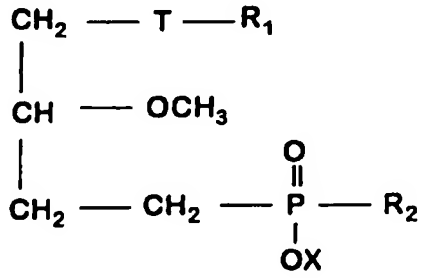
U.S. Patent No. 5,219,845, dated June 15, 1993, granted to The University of British Columbia, protects a

group of substances with a glycerol backbone linked to a phosphorus atom and a polar head group useful as anti-inflammation agents.

None of these patents discloses [4-hexadecyl-3-methoxy-butyl] phosphonic acid useful as an anti-cancer agent.

SUMMARY OF THE INVENTION

The present invention provides for an anti-cancer compound of the general formula:

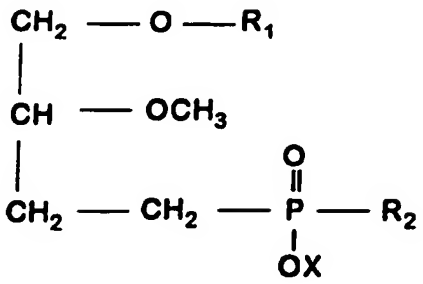


(I)

R_2 is OX or NHR

wherein T is an oxygen or sulphur atom, R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is H, or a pharmaceutically acceptable cation. The compound includes either of the opposite stereochemical configurations [(R) or (S)], or a mixture of stereoisomers.

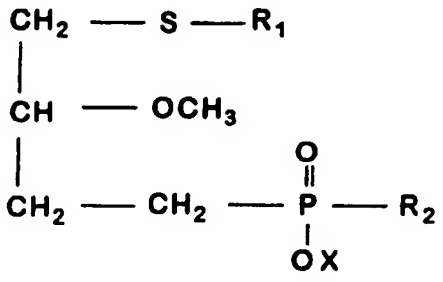
A phosphonic compound of the general formula:



(II)

wherein R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is H, or a pharmaceutically acceptable cation. The compound includes either of the opposite stereochemical configurations [(R) or (S)], or a mixture of stereoisomers.

A phosphonic compound of the general formula:

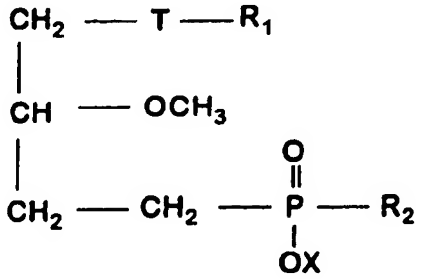


(III)

wherein R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is H, or a pharmaceutically acceptable cation. The compound includes either of the opposite stereochemical configurations [(R) or (S)], or a mixture of stereoisomers.

A method of treating cancer, lung cancer, colorectal cancer, leukemia, lymphoma or melanoma in a mammal afflicted with cancer, lung cancer, colorectal cancer, leukemia, lymphoma or melanoma, comprising treating the

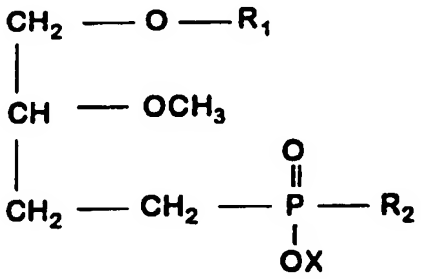
afflicted mammal with a therapeutic amount of a phosphonic compound of the following general formula:



(I)

wherein T is either an oxygen or sulphur atom, R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is H, or a pharmaceutically acceptable cation. The compound includes either of the opposite stereochemical configurations [(R) or (S)], or a mixture of stereoisomers.

A method of treating cancer of lung, colorectal cancer, leukemia, lymphoma or melanoma with a therapeutic amount of a phosphonic compound of the following formula:

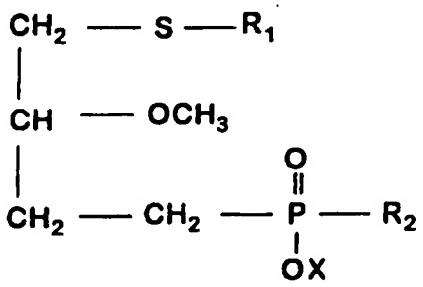


(II)

wherein R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is H, or a pharmaceutically acceptable cation. The compound includes either of the opposite stereochemical configurations [(R) or (S)], or a mixture of stereoisomers. The phosphonic compound is administered to the afflicted mammal at a dosage of 5 to 50 mg/Kg body weight, and may be adminis-

tered to the afflicted mammal orally, intravenously, intramuscularly, intradermally, subcutaneously, topically or intravenously in the form of a liposome or other lipid vesicle, with or without a pharmaceutically acceptable carrier. In the case of leukemia or lymphoma, the phosphonic compound can be administered directly to the afflicted mammal's bone marrow, blood, blood cells, leukocytes, lymphocytes or other such extracorporeal preparation containing the mammal's diseased cells, with or without a pharmaceutically acceptable carrier.

A method of treating cancer of lung, colorectal cancer, leukemia, lymphoma or melanoma in a mammal with a therapeutic amount of a phosphonic compound of the following formula:



(III)

wherein R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is H, or a pharmaceutically acceptable cation. The compound includes either of the opposite stereochemical configurations [(R) or (S)], or a mixture of stereoisomers. The phosphonic compound is administered to the afflicted mammal at a dosage of 5 to 50 mg/Kg body weight, and may be administered to the afflicted mammal orally, intravenously, intramuscularly, intradermally, subcutaneously, topically or intravenously in the form of a liposome or other lipid vesicle, with or without a pharmaceutically acceptable carrier. In the case of leukemia or lymphoma, the phosphonic acid can be administered directly to the afflicted mammal's bone marrow, blood, blood cells, leukocytes,

lymphocytes or other such extracorporeal preparation containing the mammal's diseased cells, with or without a pharmaceutically acceptable carrier.

These phosphonic compounds are useful as anti-cancer agents since they inhibit the growth of malignant cells.

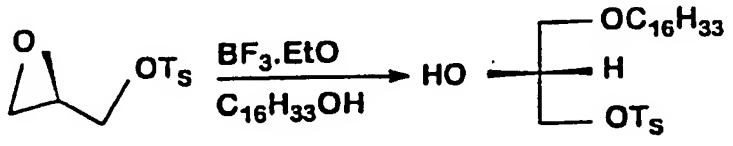
The phosphonic compounds as described above or as claimed include either of the opposite stereochemical configurations [(R) or (S)] or a mixture thereof.

DETAILED DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

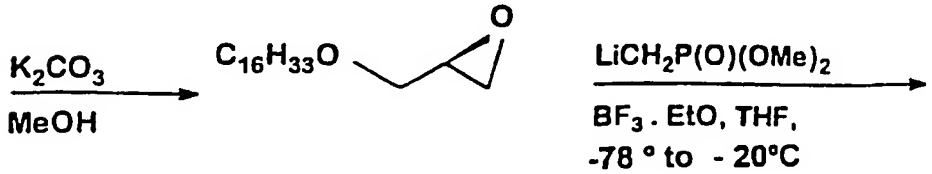
1. Production of the Compounds of the Invention

(a) Synthesis of [4-hexadecyloxy-3-methoxy-butyl] phosphonic acid

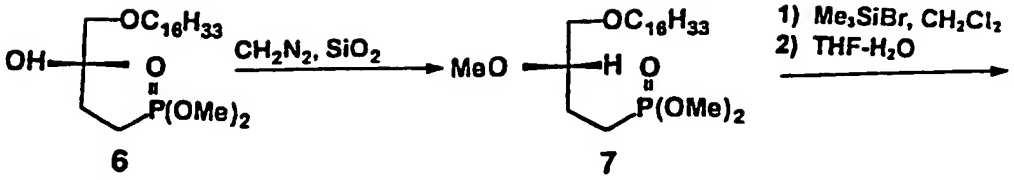
The phosphonic compounds of the invention, wherein T is oxygen, can be synthesized according to the following reaction sequence:



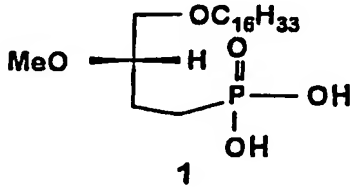
4



5



6 7



1

The reaction of 20 mmol of (S)-(+)-glycidly tosylate and 30 mmol of 1-hexadecanol in 50 ml methylene chloride in the presence of catalytic boron trifluoride etherate was carried out. After purification by flash chromatography (elution with 5:1 hexane/ethyl acetate), followed by three recrystallizations from ether-hexane, there was obtained 7.41g (80%) of ring-opened product 4 (1-*O*-hexadecyl-*sn*-glycerol 3-*O*-*p*-toluene-sulfonate).

To a suspension of 15 mmol of the tosylate 4 in 100 ml of dry methanol was added 30 mmol of powdered potassium carbonate at 0°C. The reaction mixture was stirred for 3 hours at 0°C, diluted with 300 ml of ethyl ether, and filtered through a pad of silica gel. The filtrate was concentrated under reduced pressure, and the residue was dissolved in hexane and filtered through a pad of silica gel to give 3.95g (98%) of the epoxide 5 (hexadecyl (S)-2-oxiranylmethyl ether) as a white solid, which was used without further purification.

To a solution of 40 mmol of dimethyl methanephosphonate in 30 ml of dry THF was added dropwise 40 mmol of *n*-butyllithium (a 2.5 M solution in hexane), and the reaction was stirred for 30 minutes at -78°C. To this mixture was added dropwise boron trifluoride etherate (40

mmol), followed by a solution of 20 mmol of the epoxide 5 in 100 ml of THF. The reaction mixture was stirred for 3 hours at -78°C and then warmed to -20°C and stirred for a further 1 hour. The mixture was quenched by the addition of saturated aqueous ammonium chloride solution and was concentrated under reduced pressure. The product from the aqueous residue was extracted with ether, and the combined extracts were washed with brine, dried over sodium sulfate, and concentrated under vacuum. Purification by flash chromatography on silica gel (elution with chloroform-methanol, 25:1) gave 7.54g (89%) of the product 6 (dimethyl 4-(hexadecyloxy)-3(S)-hydroxybutanephosphonate) as a white solid after lyophilization from hexane.

To a mixture of 5.0 mmol of the hydroxy phosphonate 6 and 11g of silica gel (previously heated at 150°C for 2 hours under high vacuum) was added an ether solution of diazomethane (20 molar equivalents based on substrate) at 0°C. After the mixture had stirred at 0°C for 6 hours, another 20 molar equivalents of diazomethane solution was added, and the mixture was stirred for 24 hours at 0°C. The silica gel was removed from the reaction mixture by filtration and washed with ether. The product was purified by flash column chromatography on silica gel (elution with chloroform-methanol, 50:1) to give 1.94g (88%) of the product 7 (dimethyl 4-(hexadecyloxy)-3(S)-methoxybutanephosphonate) as a colourless oil.

To a solution of 0.1 mmol of methoxy phosphonate 7 in 5 ml of methylene chloride was added 2.7 mmol of bromotrimethylsilane. After the mixture was allowed to stand for 2 hours at room temperature, volatile materials were removed under vacuum. The residue was dissolved in THF-water (17 ml, 8:1 by volume), and the mixture was allowed to stand for 2 hours at room temperature. The solvents were removed under vacuum, and the residue was

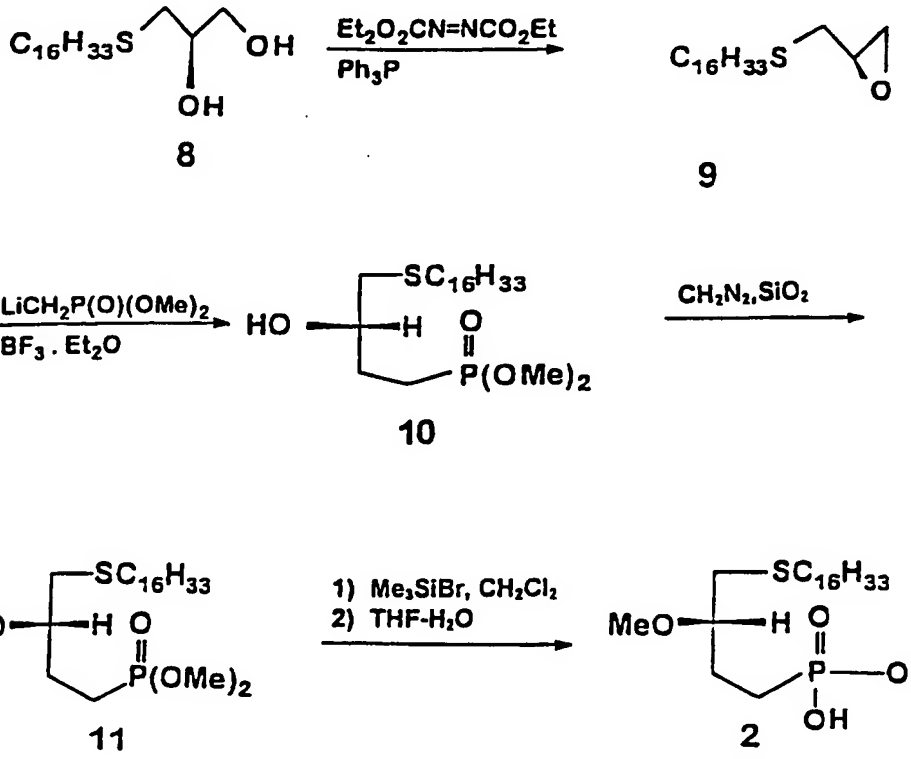
dried by repeated azeotropic distillation with dry 2-propanal under vacuum.

Lyophilization from benzene gave 408 mg (100%) of the phosphonic acid 1 ([4-hexadecyloxy-3(S)-methoxy-butyl] phosphonic acid) as a white solid.

The enantiomeric phosphonic acid ([4-hexadecyl-oxy-3(R)-methoxy-butyl] phosphonic acid) can be prepared according to the method above using the corresponding starting material.

(b) Synthesis of [4-hexadecylthio-3-methoxy-butyl] phosphonic acid

The phosphonic compounds of the invention, wherein T is sulphur, can be synthesized according to the following reaction sequence.



8 9 10 11 2

In situ $NaBH_4$-mediated opening of (S)-glycidol (prepared by asymmetric epoxidation of allyl alcohol) with hexadecyl mercaptan yielded the starting material 8 (1-(hexadecylthio)-*sn*-glycerol).

A mixture of 10 mmol of the diol 8, 15 mmol of triphenylphosphine, and 15 mmol of diethyl azodicarboxylate in 50 ml of benzene was refluxed for 24 hours. After removal of the solvent, 50 ml of ether was added, and the precipitate of phosphine oxide was removed by filtration. The filtrate was concentrated under vacuum, and the residue was purified by flash chromatography (elution with 20:1 hexane-ethyl acetate) to give 2.71g (86%) of the product 9 (hexadecyl(S)-2-oxiranylmethyl thioether) as a white solid.

To a solution of 20 mmol of dimethyl methanephosphonate in 15 ml of dry THF was added dropwise 20 mmol of *n*-butyllithium (a 2.5 M solution in hexane). After the reaction mixture was stirred for 30 minutes at -78°C, 20 mmol of boron trifluoride ethereate was added dropwise, followed by a solution of 5.0 mmol of the epoxide 9 in 50 ml of THF. The reaction mixture was stirred for 3 hours at -78°C, warmed to -20°C, stirred for 1 hour, and then quenched by the addition of saturated aqueous ammonium chloride solution. The mixture was concentrated under reduced pressure, extracted with ether, and the combined extracts were washed with brine, dried over sodium sulfate, and concentrated under vacuum. Purification by flash chromatography on silica gel (elution with chloroform-methanol, 25:1) gave 1.98g (90%) of the product 10 (dimethyl 4-(hexadecylthio)-3(S)-hydroxybutanephosphonate) as a white solid after lyophilization from hexane.

To a mixture of 5.0 mmol of the hydroxy phosponate 10 and 11g of silica gel (previously heated at 150°C for 2 hours under high vacuum) was added an ether solution

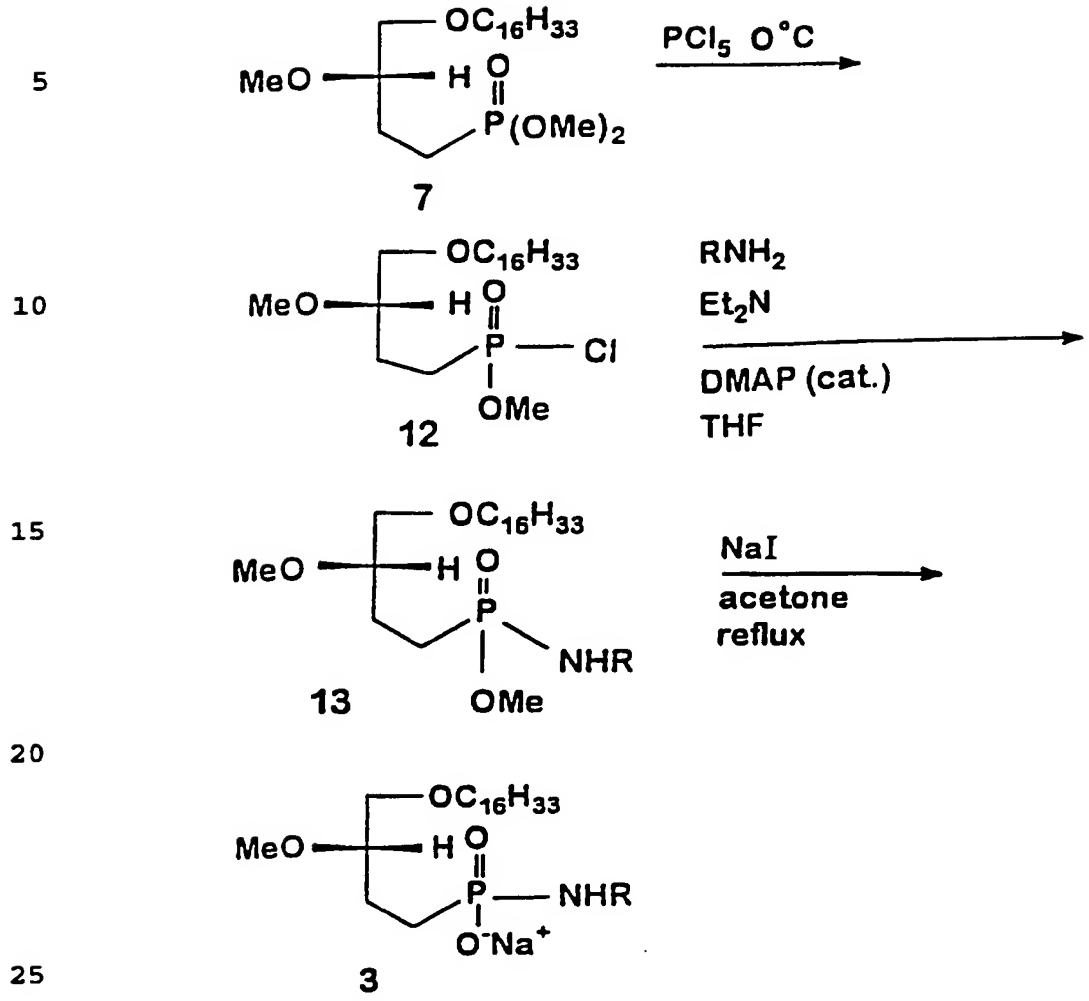
of diazomethane (20 molar equivalents based on substrate) at 0°C. After the mixture had stirred at 0°C for 6 hours, another 20 molar equivalents of diazomethane solution was added, and the mixture was stirred for 24 hours at 0°C. The silica gel was removed from the reaction mixture by filtration and washed with ether. The product was purified by flash column chromatography on silica gel (elution with chloroform-methanol, 50:1) to give 1.94g (88%) of pure product 11 (dimethyl 4-(hexadecylthio)-3(S)-methoxybutane-phosphonate) as a colourless oil.

To a solution of 0.1 mmol of methoxy phosphonate 11 in 5 ml of methylene chloride was added 2.7 mmol of bromotrimethylsilane. After the mixture was allowed to stand for 2 hours at room temperature, volatile materials were removed under vacuum. The residue was dissolved in THF-water (17 ml, 8:1 by volume), and the mixture was allowed to stand for 2 hours at room temperature. The solvents were removed under vacuum, and the residue was dried by repeated azeotropic distillation with dry 2-propanol under vacuum. Lyophilization from benzene gave the phosphonic acid 2 ([4-hexadecylthio-3(S)-methoxy-butyl] phosphonic acid) as a white solid.

The enantiomeric phosphonic acid ([4-hexadecyl-thio-3(R)-methoxy-butyl] phosphonic acid) can be prepared according to the method above using the corresponding starting material.

(c) Synthesis of protein-conjugated Phospholipid compounds

The phosphonic compounds of the invention, wherein T is oxygen or sulphur and R_2 is a protein moiety, can be synthesized according to the following reaction sequence.



The specific protein-conjugate phosphonic compound may be selected by use of the appropriate dimethyl phosphonate starting material. An example is provided here for the preparation of a [4-hexadecyloxy-3(S)-methoxybutyl] phosphonic-protein conjugate.

Dimethyl 4-(hexadecyloxy)-3(S)-methoxybutane phosphonate 7 in benzene was cooled to 0°C (ice-salt bath) and an equimolar amount of PCl_5 was added so that the

temperature did not exceed 10°C. After 1 hour of stirring, the solvent and $POCl_3$ were removed under high vacuum. The resulting acid chloride 12, was used without further purification.

To a solution of acid chloride 12 dissolved in THF was added triethylamine (1.2 equivalents) and the protein moiety (1 to 4 equivalents). This mixture was allowed to react for up to 14 hours at room temperature, catalysed by DMAP. The solvents were removed under vacuum to yield the phosphonamide 13.

To a solution of the phosphonamide 13 dissolved in acetone was added sodium iodide. The mixture was allowed to reflux for 3.5 hours, permitting the selective monodealkylation of the phosphonamide to yield the protein-conjugated phosphonic salt 3.

2. Biological Activity

In vitro tests, such as MTT assay, have been conducted to establish that phosphonic acids inhibit the growth of cancer cells and kill them. In vitro tests, as well as in vivo testing using animal models of cancer, are useful indicators of the cytotoxic activity of new anti-cancer compounds. While it would be ideal to test new compounds in human beings, such testing is unethical, and thus it is acceptable to extrapolate results of testing new anti-cancer compounds in vitro and in vivo in animal models to the human condition.

Experiments were performed using a number of different tumour cell lines. Tumor cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (50 units/ml), streptomycin (50 μg/ml) and mercaptoethanol (5 μg/ml) in an atmosphere of 5% CO_2. The cells were passaged weekly by serial 1/10 to 1/10,000

dilutions. The cell viability and growth were constantly monitored by staining with trypan blue exclusion dye or the incorporation of tritiated thymidine.

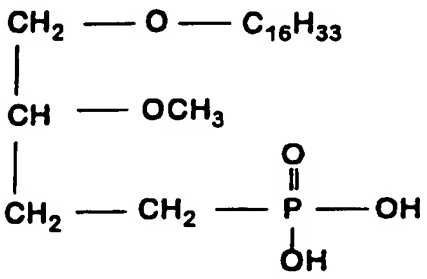
The MTT assay is performed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), a water soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by active mitochondrial dehydrogenase enzymes of living cells. Dead cells do not cause this conversion. The converted dye can be solubilized with DMSO, and the dissolved material measured spectrophotometrically, yielding absorbance as a function of concentration of converted dye. Approximately 2500 cells/well were incubated at 37°C for 24 hours in a 96-well microtiter plate. Various concentrations of the test compound (dissolved in HEPES buffer) or vehicle, diluted in 100 µL of the culture medium, or culture medium alone were added to each well, and the cells incubated for a further 24 to 72 hours. MTT working solution (50 µL of a 1:5 (v/v) diluted stock solution prepared as per the manufacturer's directions) was added to each well and the cells incubated at 37°C for 4 hours. The culture supernatant was aspirated, leaving 10 to 20 µL in the bottom of each well, and 150 µL DMSO was added to solubilize any converted dye. The absorbance in each well was read in a spectrophotometer at 540 nm, and the cell viability in the wells containing the test compounds expressed as a percentage of the absorbance in control wells.

(a) **Activity of phosphonic acids against colorectal cancer**

Example 1

Activity of Phosphonic Acids Against Human Colorectal Cancer Cells

It has been discovered that the phosphonic acid of the following formula:



(IV)

has a cytotoxic effect against HT-29 cells (human colorectal cancer) in the MTT cell viability assay when the compound is administered either as the sodium salt or the free acid. This effect is indicative of the anti-cancer activity of the above phosphonic acid (compound IV).

Table 1 shows the effect of 72 hours' exposure to various concentrations of the phosphonic acid of the above formula (compound IV) on the viability of HT-29 cells.

Table 1

MTT Cell Viability Assay of HT-29 Tumor Cells Exposed to Various Concentrations of Phosphonic Acid (Compound IV) for 72 Hours

Phosphonic acid concentration (μM)	Mean (n = 3) Absorbance (% of control)
0.0	100.00
0.1	99.97
0.5	89.21
1.0	77.29
2.5	62.20
5.0	56.71
10.0	35.50
25.0	12.98
50.0	9.14

Example 2

Table 2 shows the results of an assay performed to observe the effects of compound IV (phosphonic acid) on H460 (human cell lung cancer) cells. Table 2 shows the effect of 72 hours' exposure to various concentrations of the phosphonic acid of the above formula (compound IV) on the viability of H460 cells.

Table 2

MTT Cell Viability Assay of H460 Tumor Cells Exposed to Various Concentrations of Phosphonic Acid (Compound IV) for 72 Hours

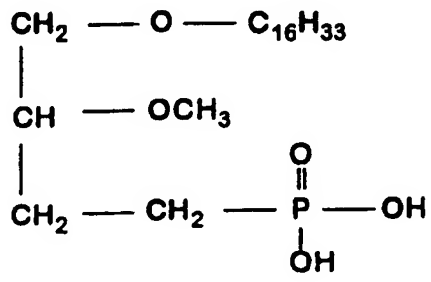
Phosphonic acid (Na salt) concentration (μM)	Mean (n = 3) Absorbance (% of control)
0.0	100
	100
0.2	96
0.4	88
0.8	78
1.6	47
3.2	12
6.4	0
12.8	0

As can be seen from Tables 1 and 2, phosphonic acids compounds of the formula shown above (compound IV) are cytotoxic against H460 human colorectal cancer cells and lung cancer cells over 72 hours when administered as the sodium salt. This effect is dose-dependent, and demonstrates the anti-cancer activity of phosphonic acids.

Example 3

Activity of Phosphonic Acids Against Murine Metastatic Colon Cancer Cells

It has been discovered that the phosponic acid compound of the following formula:



(IV)

has a cytotoxic effect against metastatic Colon 26 cells (murine colon cancer) in the MTT cell viability assay when the compound is administered as the sodium salt. This effect is indicative of the anti-cancer activity of the above phosphonic acid (compound IV).

Table 3 shows the effect of 72 hours' exposure to various concentrations of the phosphonic acid of the above formula (compound IV) on the viability of metastatic Colon 26 cells.

Table 3

MTT Cell Viability Assay of Metastatic Colon 26 Tumor Cells Exposed to Various Concentrations of Phosphonic Acid (Compound IV) for 72 Hours

Phosphonic acid (Na salt) concentration (μM)	Mean (n = 3) Absorbance (% of control)
0.0	100.00
0.1	106.91
0.5	101.20
1.0	105.21
2.5	105.17
5.0	87.32
10.0	61.46
25.0	18.01
50.0	0.46

As can be seen from Table 3, phosphonic acids of the formula shown above (compound IV) are cytotoxic against Colon 26 murine metastatic colon cancer cells over 72 hours when administered as the sodium salt. This effect is dose-dependent, and demonstrates the anti-cancer activity of phosphonic acids.

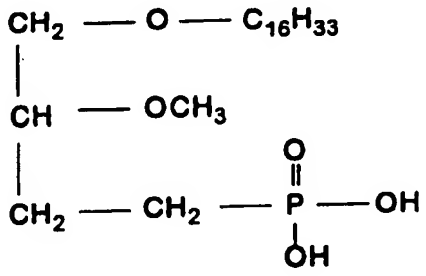
Examples 1 and 2 clearly demonstrate that the phosphonic compounds of the invention have a cytotoxic action against colorectal cancer, and are thus useful as anti-cancer agents.

(b) **Activity of Phosphonic Acids Against Leukemia and Lymphoma**

Example 4

Activity of Phosphonic Acids Against a Human Myeloleukemic Cell Line

It has been discovered that the phosphonic acid of the following formula:



(IV)

has a cytotoxic effect against HL-60 tumor cells (a human myeloleukemic cell line) in the MTT cell viability assay when the compound is administered as the sodium salt. This effect is indicative of the anti-cancer activity of the above phosphonic acid (compound IV).

Table 4 shows the effect of 72 hours' exposure to various concentrations of the phosphonic acid of the above formula (compound IV) on the viability of HL-60 cells.

Table 4

MTT Cell Viability Assay of HL-60 Tumor Cells Exposed to Various Concentrations of Phosphonic Acid (Compound IV) for 72 Hours

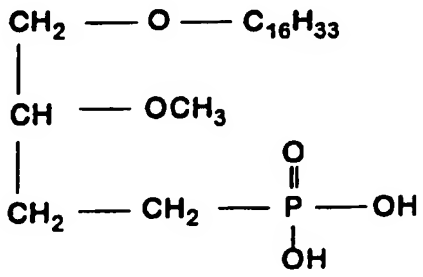
Phosphonic acid (Na salt) concentration (μM)	Mean (n = 3) Absorbance (% of control)
0.0	100
0.1	95
0.2	93
0.4	88
0.8	80
1.6	61
3.2	40
6.4	6
12.8	0

As can be seen from Table 4, phosphonic acid compounds of the formula shown above (compound IV) are cytotoxic against HL-60 human myeloleukemic cells over 72 hours when administered as the sodium salt. This effect is dose-dependent, and demonstrates the anti-cancer activity of phosphonic acids.

Example 5

Activity of Phosphonic Acids Against Mouse Lymphoma

It has been discovered that the phosphonic acid of the following formula:



(IV)

has a cytotoxic effect against L1210 cells (murine lymphoma) in the MTT cell viability assay when the compound is administered as the sodium salt. This effect is indicative of the anti-cancer activity of the above phosphonic acid (compound IV).

Table 5 shows the effect of 72 hours' exposure to various concentrations of the phosphonic acid of the above formula (compound IV) on the viability of L1210 cells.

Table 5

MTT Cell Viability Assay of L1210 Tumor Cells Exposed to Various Concentrations of Phosphonic Acid (Compound IV) for 72 Hours

Phosphonic acid (Na salt) concentration (μM)	Mean (n = 3) Absorbance (% of control)
0.0	100
0.1	95
0.2	81
0.4	87
0.8	78
1.6	62
3.2	31
6.4	3
12.8	0

As can be seen from Table 5, phosphonic acid compounds of the formula shown above (compound IV) are cytotoxic against L1210 murine lymphoma over 72 hours when administered as the sodium salt. This effect is dose-dependent, and demonstrates the anti-cancer activity of phosphonic acids.

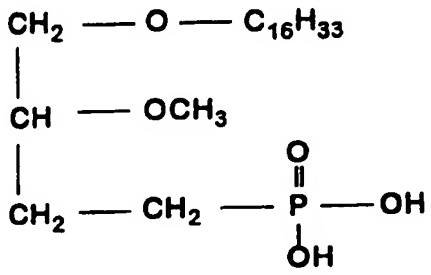
Examples 4 and 5 clearly demonstrate that the phosphonic compounds of the invention have a cytotoxic action against leukemia and lymphoma, and are thus useful as anti-cancer agents.

(c) Activity of Phosphonic Acids Against Melanoma

Example 6

Activity of Phosphonic Acids Against Human Melanoma

It has been discovered that the phosphonic acid compound of the following formula:



(IV)

has a cytotoxic effect against RPMI 7951 cells (human melanoma cell line) in the MTT cell viability assay when the compound is administered as the sodium salt. This effect is indicative of the anti-cancer activity of the above phosphonic acid (compound IV).

Table 6 shows the effect of 72 hours' exposure to various concentrations of the phosphonic acid of the above formula (compound IV) on the viability of RPMI 7951 cells.

Table 6

MTT Cell Viability Assay of RPMI 7951 Tumor Cells Exposed to Various Concentrations of Phosphonic Acid (Compound IV) for 72 Hours

Phosphonic acid (Na salt) concentration (μM)	Mean (n = 3) Absorbance (% of control)
0.0	100
0.1	91
0.2	89
0.4	88
0.8	74
1.6	67
3.2	52
6.4	37
12.8	7

As can be seen from Table 6, phosphonic acids of the formula shown above (compound IV) are cytotoxic against RPMI 7951 human melanoma cells over 72 hours when administered as the sodium salt. This effect is dose-dependent, and demonstrates the anti-cancer activity of phosphonic acids.

Example 5 clearly demonstrates that the phosphonic compounds of the invention have a cytotoxic action against human melanoma, and are thus useful as anti-cancer agents.

In addition to the cytotoxicity, compound IV showed to be a potent inhibitor of phosphatidylinositol 3 Kinase (PI_3Kinase) as shown in Table 7.

Table 7

Effect of Compound IV on the Activity of PI_3Kinase as Determined by Incorporation of ^{32}P to PIP_2

Compound IV concentration(μM)	PI_3Kinase activity (^{32}P CPm)
Control	4105±205
20	427±28
200	32±7

Inhibitory constants: $1C_{50}$ = 10μM; Ki - 2 μM

The PI_3Kinase activity is determined as the incorporation of ^{32}P at the 3-position of the inositol ring of phosphatidylinositol (PI) is in the presence of ^{32}P-ATP.

3. Usage and Dosage

The compounds of the invention are useful as anti-cancer agents, and may be administered safely by either parenteral, oral or topical routes in pharmaceutical preparations such as injections, tablets, capsules, liquid preparations or ointments. These preparations are used by an appropriate route of administration, depending on the specific affliction, patient conditions and other factors. Injections may be given intravenously, intramuscularly, intradermally or subcutaneously. The dose of compound can be selected based on the patient weight, treatment regimen or purpose of administration, generally within the range of 5 to 50 mg/Kg. These compound preparations may be administered 1 to 4 times daily, at 2 to 7 day intervals, or as otherwise necessary to maintain a therapeutic level of the compound in body tissues depending on the specific affliction, patient conditions, treatment regimen or purpose of administration.

Injections, intravenous infusions and similar preparations are prepared by conventional methods in either aqueous solution or physiological saline containing 20% propylene glycol and a preservative such as 0.5% ascorbic acid, with an upwardly adjusted pH in phosphate buffer. The drug solution is sterilized by passing it through a 22 μm filter, and distributed into glass vials in approximately 1 ml aliquots to provide a unit dosage. The aliquots are then lyophilized, and the vials tightly stoppered and capped to maintain sterility. The drug may be reconstituted in the vial by the addition of physiological saline or aqueous diluent.

Tablets are prepared by conventional methods. Unit dosage tablets can be prepared by compressing a mixture of 40 mg of phosphonic acid compound, 200 mg of lactose, and 50 mg Avicel™ into the form of a tablet. A similar drug mixture may also be contained in unit dose within a cellulose-based capsule.

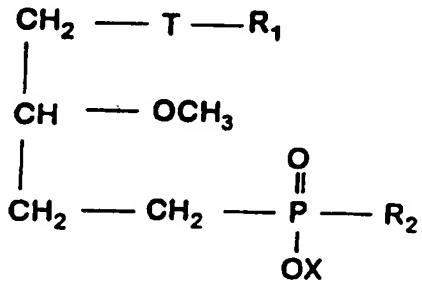
An ointment or cream may also be prepared by conventional methods by mixing the phosphonic acid compound in a commercially-available glycerine-based cream. The cream is applied topically directly to the afflicted area.

The compounds of the invention may also be administered in the form of a liposome. A mixture of phosphonic acid compound and lecithin is mechanically treated to form a bilayer (one side hydrophilic, the other hydrophobic) which spontaneously forms micelles (liposomes). These may be filtered to obtain liposomes of uniform size (approximately 10 nm) and dose (approximately 50 mg/L). Liposomes are sterilized by filtration through a 22 μm filter, and administered as an intravenous solution.

As will be apparent to those skilled in the art in the light of the foregoing disclosure, many alterations and modifications are possible in the practice of this invention without departing from the spirit or scope thereof. Accordingly, the scope of the invention is to be construed in accordance with the substance defined by the following claims.

WHAT IS CLAIMED IS:

1. A method of treating cancer in a mammal afflicted with cancer, comprising treating the afflicted mammal with a therapeutic amount of a phosphonic compound of the formula:



wherein T is an oxygen or sulphur atom, R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is a hydrogen atom, or a pharmaceutically acceptable cation, and either of the opposite stereochemical configurations [(R) or (S)], or a mixture of stereoisomers thereof, and pharmaceutically acceptable salts thereof.

2. A method as claimed in claim 1, wherein T is an oxygen atom, R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is a hydrogen atom, or a pharmaceutically acceptable cation.

3. A method as claimed in claim 1, wherein T is an oxygen atom, R_1 is an aliphatic chain containing 12 to 20 carbon atoms and 33 hydrogen atoms and R_2 is a protein moiety, or OX where X is a hydrogen atom, or a pharmaceutically acceptable cation, for treatment of colorectal, and lung cancers, or lymphoma, leukemia or melanoma.

4. A method as claimed in claim 1, wherein T is an oxygen atom, R_1 is an aliphatic chain containing 16 carbon

atoms and 33 hydrogen atoms and R_2 is a protein moiety, or OX where X is a hydrogen atom, or a pharmaceutically acceptable cation.

5. A method as claimed in claim 1, wherein T is a sulphur atom, R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is a hydrogen atom, or a pharmaceutically acceptable cation.

6. A method as claimed in claim 1, wherein T is a sulphur atom, R_1 is an aliphatic chain containing 16 carbon atoms and 33 hydrogen atoms and R_2 is OX where X is a hydrogen atom, or a pharmaceutically acceptable cation.

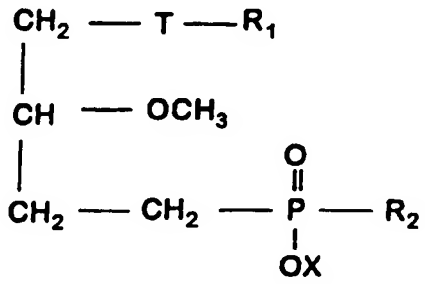
7. A method as claimed in claim 1, wherein T is a sulphur atom, R_1 is an aliphatic chain containing 16 carbon atoms and 33 hydrogen atoms, R_2 is a protein moiety, and X is a hydrogen atom, or a pharmaceutically acceptable cation.

8. A method as claimed in claim 1, wherein the protein moiety is an antibody targeted to a specific antigen or other cellular marker, used in the treatment of cancer.

9. A method as claimed in claim 1, wherein the phosphonic compound, or a pharmaceutically acceptable acid or salt thereof, is administered to the afflicted mammal orally, intravenously, intramuscularly, intradermally, subcutaneously, topically or intravenously in the form of a liposome or other lipid vesicle, with or without a pharmaceutically acceptable carrier.

10. A method as claimed in claim 1, wherein the phosphonic compound is administered to the afflicted mammal at a dosage of 5 to 50 mg/kg body weight.

11. A method of treating colorectal cancer in a mammal afflicted with colorectal cancer, comprising treating the afflicted mammal with a therapeutic amount of a phosphonic compound of the formula:



wherein T is an oxygen or sulphur atom, R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is a hydrogen atom, or a pharmaceutically acceptable cation, and either of the opposite stereochemical configurations [(R) or (S)], or a mixture of stereoisomers thereof, and pharmaceutically acceptable salts thereof.

12. A method as claimed in claim 11, wherein T is an oxygen atom, R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is a hydrogen atom, or a pharmaceutically acceptable cation.

13. A method as claimed in claim 11, wherein T is an oxygen atom, R_1 is an aliphatic chain containing 16 carbon atoms and 33 hydrogen atoms and R_2 is OX where X is a hydrogen atom, or a pharmaceutically acceptable cation.

14. A method as claimed in claim 11, wherein T is an oxygen atom, R_1 is an aliphatic chain containing 16 carbon atoms and 33 hydrogen atoms and R_2 is a protein moiety, and X is a hydrogen atom, or a pharmaceutically acceptable cation.

15. A method as claimed in claim 11, wherein T is a sulphur atom, R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is a hydrogen atom, or a pharmaceutically acceptable cation.

16. A method as claimed in claim 11, wherein T is a sulphur atom, R_1 is an aliphatic chain containing 16 carbon atoms and 33 hydrogen atoms and R_2 is OX where X is a hydrogen atom, or a pharmaceutically acceptable cation.

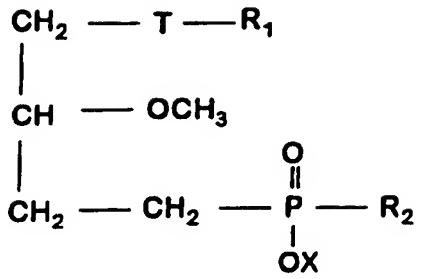
17. A method as claimed in claim 11, wherein T is a sulphur atom, R_1 is an aliphatic chain containing 16 carbon atoms and 33 hydrogen atoms, R_2 is a protein moiety, and X is a hydrogen atom, or a pharmaceutically acceptable cation.

18. A method as claimed in claim 11, wherein the protein moiety is an antibody targeted to a specific antigen or other cellular marker, used in the treatment of colorectal cancer.

19. A method as claimed in claim 11, wherein the phosphonic compound, or a pharmaceutically acceptable acid or salt thereof, is administered to the afflicted mammal orally, intravenously, intramuscularly, intradermally, subcutaneously, topically or intravenously in the form of a liposome or other lipid vesicle, with or without a pharmaceutically acceptable carrier.

20. A method as claimed in claim 11, wherein the phosphonic compound is administered to the afflicted mammal at a dosage of 5 to 50 mg/kg body weight.

21. A method of treating leukemia or lymphoma, melanoma or lung cancer in a mammal afflicted with these cancers, comprising treating the afflicted mammal with a therapeutic amount of a phosphonic compound of the formula:



wherein T is an oxygen or sulphur atom, R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is a hydrogen atom, or a pharmaceutically acceptable cation, and either of the opposite stereochemical configurations [(R) or (S)], or a mixture of stereoisomers thereof, and pharmaceutically acceptable salts thereof.

22. A method as claimed in claim 21, wherein T is an oxygen atom, R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is a hydrogen atom, or a pharmaceutically acceptable cation.

23. A method as claimed in claim 21, wherein T is an oxygen atom, R_1 is an aliphatic chain containing 16 carbon atoms and 33 hydrogen atoms, and R_2 is OX where X is a hydrogen atom, or a pharmaceutically acceptable cation.

24. A method as claimed in claim 21, wherein T is an oxygen atom, R_1 is an aliphatic chain containing 16 carbon atoms and 33 hydrogen atoms, R_2 is a protein moiety, and X is a hydrogen atom, or a pharmaceutically acceptable cation.

25. A method as claimed in claim 21, wherein T is a sulphur atom, R_1 is an aliphatic chain containing 12 to 20

carbon atoms and R_2 is a protein moiety, or OX where X is a hydrogen atom, or a pharmaceutically acceptable cation.

26. A method as claimed in claim 21, wherein T is a sulphur atom, R_1 is an aliphatic chain containing 16 carbon atoms and 33 hydrogen atoms and R_2 is OX where X is a hydrogen atom, or a pharmaceutically acceptable cation.

27. A method as claimed in claim 21, wherein T is a sulphur atom, R_1 is an aliphatic chain containing 16 carbon atoms and 33 hydrogen atoms, R_2 is a protein moiety, and X is a hydrogen atom, or a pharmaceutically acceptable cation.

28. A method as claimed in claim 21, wherein the protein moiety is an antibody targeted to a specific antigen or other cellular marker, used in the treatment of leukemia or lymphoma.

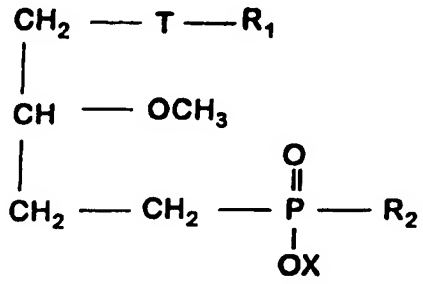
29. A method as claimed in claim 21, wherein the phosphonic compound, or a pharmaceutically acceptable acid or salt thereof, is administered to the afflicted mammal orally, intravenously, intramuscularly, intradermally, subcutaneously, topically or intravenously in the form of a liposome or other lipid vesicle, with or without a pharmaceutically acceptable carrier.

30. A method as claimed in claim 21, wherein the phosphonic compound is administered to the afflicted mammal at a dosage of 5 to 50 mg/kg body weight.

31. A method as claimed in claim 21, wherein the phosphonic compound, or a pharmaceutically acceptable acid or salt thereof, is administered directly to the afflicted mammal's bone marrow, blood, blood cells, leukocytes, lymphocytes or other such extracorporeal preparations

containing the mammal's diseased cells, with or without a pharmaceutically acceptable carrier.

32. A method of treating melanoma in a mammal afflicted with melanoma, comprising treating the afflicted mammal with a therapeutic amount of a phosphonic compound of the formula:



wherein T is an oxygen atom, R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is a hydrogen atom, or a pharmaceutically acceptable cation, and either of the opposite stereochemical configurations [(R) or (S)], or a mixture of stereoisomers thereof, or a pharmaceutically acceptable salt thereof.

33. A method as claimed in claim 32, wherein T is an oxygen atom, R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is a hydrogen atom, or a pharmaceutically acceptable cation.

34. A method as claimed in claim 32, wherein T is an oxygen atom, R_1 is an aliphatic chain containing 16 carbon atoms and 33 hydrogen atoms, and R_2 is OX where X is a hydrogen atom, or a pharmaceutically acceptable cation.

35. A method as claimed in claim 32, wherein T is an oxygen atom, R_1 is an aliphatic chain containing 16 carbon atoms and 33 hydrogen atoms, R_2 is a protein moiety, and X

is a hydrogen atom, or a pharmaceutically acceptable cation.

36. A method as claimed in claim 32, wherein T is a sulphur atom, R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is a hydrogen atom, or a pharmaceutically acceptable cation.

37. A method as claimed in claim 32, wherein T is a sulphur atom, R_1 is an aliphatic chain containing 16 carbon atoms and 33 hydrogen atoms and R_2 is OX where X is a hydrogen atom, or a pharmaceutically acceptable cation.

38. A method as claimed in claim 32, wherein T is a sulphur atom, R_1 is an aliphatic chain containing 16 carbon atoms and 33 hydrogen atoms, R_2 is a protein moiety, and X is a hydrogen atom, or a pharmaceutically acceptable cation.

39. A method as claimed in claim 32, wherein the protein moiety is an antibody targeted to a specific antigen or other cellular marker, used in the treatment of melanoma.

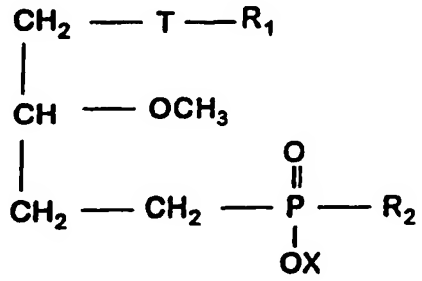
40. A method as claimed in claim 32, wherein the phosphonic compound, or a pharmaceutically acceptable acid or salt thereof, is administered to the afflicted mammal orally, intravenously, intramuscularly, intradermally, subcutaneously, topically or intravenously in the form of a liposome or other lipid vesicle, with or without a pharmaceutically acceptable carrier.

41. A method as claimed in claim 32, wherein the phosphonic compound is administered to the afflicted mammal at a dosage of 5 to 50 mg/kg body weight.

42. A method as claimed in claim 1, wherein the phosphonic compound includes either of the opposite stereochemical configurations [(R) or (S)], or a mixture of stereoisomers.

43. The compound as claimed in claim 1 used as inhibitor of PI_3Kinase, and the biological systems that this enzyme is used to prevent a symptom.

44. A phosphonic compound of the formula:



wherein T is an oxygen or sulphur atom, R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is a hydrogen atom, or a pharmaceutically acceptable cation, and either of the opposite stereochemical configurations [(R) or (S)], or a mixture of stereoisomers thereof, and pharmaceutically acceptable salts thereof.

45. A compound as claimed in claim 44, wherein T is an oxygen atom, R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is a hydrogen atom, or a pharmaceutically acceptable cation.

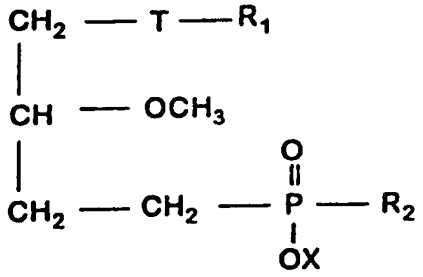
46. A compound as claimed in claim 44, wherein T is an oxygen atom, R_1 is an aliphatic chain containing 16 carbon atoms and 33 hydrogen atoms and R_2 is a protein moiety, or OX where X is a hydrogen atom, or a pharmaceuti-

cally acceptable cation, for treatment of colorectal, and lung cancers, or lymphoma, leukemia or melanoma.

47. A compound as claimed in claim 46 wherein R_2 is OH and OX is OH.

48. A compound as claimed in claim 44 wherein R is NHR.

49. The use of a compound of the formula:



wherein T is an oxygen or sulphur atom, R_1 is an aliphatic chain containing 12 to 20 carbon atoms and R_2 is a protein moiety, or OX where X is a hydrogen atom, or a pharmaceutically acceptable cation, and either of the opposite stereochemical configurations [(R) or (S)], or a mixture of stereoisomers thereof, and pharmaceutically acceptable salts thereof, in the treatment of cancer in a mammal afflicted with cancer, comprising administering to the afflicted mammal a therapeutic amount of the compound of the formula.

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/CA 95/00068

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07F9/38 A61K47/48 C07K14/00 A61K31/66 A61K38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07F A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US,A,5 219 845 (HASSAN SALARI) 15 June 1993 cited in the application	1-49
X	see column 9, line 20 - line 35	44
Y	TETRAHEDRON LETT. (TELEAY,00404039);93; VOL.34 (22); PP.3539-42, UNIV. RHODE ISLAND;DEP. CHEM.; KINGSTON; 02881; RI; USA (US) Li Z et al 'Phosphonate isosteres of phospholipids'	1-49
X	see page 3540, scheme 2, compound 15 b	44
A	EP,A,0 230 575 (MAX-PLANCK GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V.) 5 August 1987 cited in the application see the whole document	1-49

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
22 September 1995	25.10.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Beslier, L

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA95/00068

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-42
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1 to 42 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/CA 95/00068

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-5219845	15-06-93	US-A-	5369097	29-11-94
		WO-A-	9219627	12-11-92
		EP-A-	0581793	09-02-94
EP-A-0230575	05-08-87	DE-A-	3641379	03-09-87
		DE-A-	3641491	17-09-87
		DE-A-	3685214	11-06-92
		WO-A-	8703480	18-06-87
		WO-A-	8703478	18-06-87
		EP-A-	0225608	16-06-87
		EP-A,B	0248047	09-12-87
		EP-A,B	0248062	09-12-87
		IE-B-	59777	06-04-94
		IE-B-	59778	06-04-94
		JP-T-	63501874	28-07-88
		JP-T-	63501719	14-07-88
		NO-B-	174877	18-04-94
		NO-B-	175620	01-08-94
		US-A-	4837023	06-06-89
		US-A-	5049552	17-09-91